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(54) Abstract Title
Use of a PPAR gamma agonist in the production of a medicament for the prevention or treatment of diseases associated with IL-10 production

(57) The known peroxisome proliferator activated receptor gamma (PPARγ) agonists rosiglitazone, pioglitazone, troglitazone and KRP-297 have been found to inhibit Interleukin-10 (IL-10) production. Such compounds find use in the prevention or treatment the following diseases - systemic lupus erythematosus, infectious diseases, AIDS (especially related lymphomas), cancer, transplantation, burn-injury-induced immune suppression, psoriasis, rheumatoid arthritis, diseases where high Th1 responses are therapeutic and allergic diseases.

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Organic Compounds

The present invention relates to organic compounds, e.g. PPARγ agonists, e.g. including thiazolidinediones.

One mechanism through which thiazolidinediones are believed to have biological effect is their ability to serve as a high affinity ligand for the orphan steroid receptor peroxisome proliferator-activated receptor gamma (PPARγ) (Lehmann et al, J. Biol. Chem., 270:12953-12956, 1995.). PPARγ is a member of the nuclear receptor superfamily of orphan steroid receptors that serve as transcription factors (Motojima, Cell Structure and Function, 18:267-277, 1993). This family includes receptors for the steroid, thyroid and retinoid hormones. Activation of PPARγ is implicated in adipocyte differentiation through the activation of adipocyte-specific gene expression (Lehmann et al.,1995, ibidem). This gene expression is mediated through binding to a PPARγ response element (PPRE) in the promoter region of target genes (Forman et al, Cell, 83:803-812, 1995.). This PPRE is composed of a directly repeating core site separated by one nucleotide. To bind to a PPRE, PPARγ must form a heterodimer with the 9-cis retinoic acid receptor (RXR). This sequence is classified as a DR-1 consensus sequence that is universal for orphan receptors (Vidal-Puig et al, J. Clin. Invest., 97:2553-2561, 1996). Because of the universal nature of this consensus sequence, other transcription factors can bind to the PPRE and compete with the binding of PPARγ. One such transcription factor is COUP-TFII that antagonizes PPAR signaling in mammalian cells (Marcus et al., Mol. Cell. Endocrinol., 120:31-39, 1996). PPAR is in a family of three orphan receptors that are encoded by different genes (Motojima, 1993, ibidem). The three PPAR genes are PPARα, PPARδ, and PPARγ (Motojima, 1993, ibidem). In addition to thiazolidinediones, another ligand for PPARγ nuclear receptor is the arachidonic acid metabolite 15-deoxy-delta^{12,14}-prostaglandin J_2 (15d-PGJ_2). This prostaglandin activates PPARγ-dependent adipogenesis, but activates PPARα only at high concentrations (Forman et al., 1995, ibidem; Kliewer et al., Cell, 83:813-819, 1995. 1995). This is further evidence that the PPAR family subtypes are distinct from one another in their pharmacological response to ligands. PPARs have a function in ligand-activated transcription and have been found to control glucose and lipid metabolism.

Compounds which influence the action of PPARγ (PPARγ-agonists), e.g. including compounds which bind to PPARγ, are known or may be found according to an appropriate

method, e.g. by determining the binding activity of a compound to PPARγ, e.g. by use of an appropriate assay, e.g. including a PPARγ assay according to a method as conventional. Some known PPARγ-agonists are therapeutically useful e.g. in the treatment of diabetes, e.g. having hypolipidemic and hypoglycemic activities, improving the insulin resistance, lowering blood sugar and blood lipid levels and in the treatment of impaired glucose tolerance to preventing or delay the onset of noninsulin-dependent diabetes mellitus.

Known PPARγ-agonists e.g. include compounds as disclosed in WO91/07107; WO 92/02520; WO 94/01433; WO 89/08651; US 4,287,200; US 4,340,605; US 4,438,141; US 4,444,779; US 4,461,902; US 4,572,912; US 4,687,777; US 4,703,052; US 4,725,610; US 4,873,255; US 4,897,393; US 4,897,405; US 4,918,091; US 4,948,900; US 5,002,953; US 5,061,717; US 5,120,754; US 5,132,317; US 5,194,443; US 5,223,522; US 5,232,925; US 5,260,445. Specifically troglitazone and pioglitazone are disclosed in EP-0 008 203, EP-0 139 421 and EP-0 193 256, rosiglitazone in EP-0 306 228 and the compound KRP-297 in EP 846 693. The use of such compounds in the treatment of impaired glucose tolerance to prevent or delay the onset of noninsulin-dependent diabetes mellitus is e.g. disclosed in WO 95/07649. The disclosure of these publications is incorporated herein by reference in particular with respect to the active compounds disclosed therein, and methods of preparation thereof.

Interleukin 10 (IL-10), a cytokine produced by T lymphocytes, was first identified by its ability to inhibit interferon gamma (IFN-γ) and IL-2 synthesis by mouse and human T lymphocytes (Fiorentino et al., 1989, J. Exp. Med. 170:2081-2089; Moore et al., 1990, Science 248:1230-1252; Vieira et al., 1991, Proc. Natl. Acad. Sci. USA 88:1172-1177). IL-10 can be produced by many kinds of cells including Th2 cells, macrophages, monocytes, mast cells and B cells (Moore K. W. et al 1993, Ann. Rev. Immunol. 11, 165; MacNeil I. et al, J. Immunol. 145, 4167 (1990); Thompson-Snipes L. et al, J. Exp. Med. 173, 507. 8-11 (1991)). IL-10 exerts a wide range of effects on a variety of cell types. IL-10 regulates the synthesis of a wide spectrum of cytokines produced by T cells and monocytes.
Macrophages are cells of the innate immune system and are a major source of cytokines. During the onset of acute inflammation and in the progress of chronic inflammation, cytokines play a pivotal role. Interleukin-10 (IL-10) is usually described as an antiinflammatory cytokine because it can attenuate the inflammatory response by inhibiting the release of proinflammatory cytokines. However, an overexpression of IL-10 is seen in a

number of immune-related disorders, such as systemic lupus erythematosus, see e.g. Ishida et al, J Exp Med 1994;179:305-10 (1994), al-Janadi et al, 1996, J.Clin.Immunol. 16: 198-207; Kalechman et al, J Immunol 1997;159:2658-2667; Llorente et al, Arthritis Rheum 2000; 43:1790-1800.

A number of human monocytic cell lines have been explored as model systems for studying lipopolysaccharide (LPS)-induced cytokine production. One of the more commonly used is the well-characterized U937 cell line (Sundstrom, C, Nilsson, Int J Cancer 1976;17:565-577, 1976). The LPS induction of cytokines, such as TNF-α, IL-10, IL-6, IL-8, IL-1beta, and IL-1 receptor antagonist (IL-1ra), has been studied in this system (Prehn et al, Blood 1992; 80:2811-2816; Takeshita et al, J Immunol 1996;156:2591-2598; Kavelaars et al, J Neuroimmunol 1997; 77:211-216; Mander et al, Int J Immunopharmac 1997;19:451-462; Roberts et al, Infect Immun 1997;65:3248-3254; Izeboud et al, J Recept Sig Trans Res 1999;19:191-202). In the present study, as also described in the Example, U937 cells were differentiated into a macrophage-like phenotype with phorbol ester and then stimulated with LPS, in order to induce the expression of IL-10. Known agonists of PPARγ, e.g. including troglitazone pioglitazone, rosiglitazone and the compound KRP-297, have been shown to inhibit LPS-stimulated IL-10 secretion.

We have now found that PPARγ-agonists, e.g. including such as cited above, e.g. such as cited in the patent filings above, e.g. including troglitazone, pioglitazone, rosiglitazone and the compound KRP-297, may influence, e.g. inhibit, the production of IL-10.

The inhibition of IL-10 secretion by PPARγ agonists according to the present invention, e.g. as shown in the Example, suggests new, additional therapeutic applications for PPARγ agonists. These include antibody-mediated autoimmune diseases, such as systemic lupus erythematosus in which IL-10 is elevated (Ishida et al, 1994, ibidem; al-Janadi et al, 1996, ibidem; Kalechman et al, 1997, ibidem; Llorente et al, 2000, ibidem), as well as other disease states, where IL-10 might play a deleterious role, such as infectious diseases (Bermudez, et al, "Infection with Mycobacterium avium induces production of interleukin-10 (IL-10), and administration of anti-IL-10 antibody is associated with enhanced resistance to infection in mice", Infect Immun 1993: 61:3093-3097; Swierczynski B. et al, "Inhibitory activity of anti-interleukin-4 and anti-interleukin-10 antibodies on Toxoplasma gondii proliferation in mouse peritoneal macrophages cocultured with splenocytes from infected mice", Parasitology Res 2000;86:151-157; cancer (Peng et al, "Growth inhibition of malignant CD5+B (B-1) cells by

antisense IL-10 oligonucleotide", Leuk Res 1995; 19: 159-67; Yue et al, "Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-I, HLA class-II and ICAM-1 molecules", Int. J. Cancer 1997; 71: 630-637; Kim et al "Inhibition of interleukin-10 (IL-10) production from MOPC 315 tumor cells by IL-10 antisense oligodeoxynucleotides enhances cell-mediated immune responses", Cancer Immunol Immunother 2000; 49: 433-440), AIDS and AIDS-related lymphomas (Masood et al, "Interleukin-10 is an autocrine growth factor for acquired immunodeficiency syndrome-related B-cell lymphoma", Blood 1995; 85: 3423-3430; Doherty et al, "Modulation of murine AIDS-related pathology by concurrent antibody treatment and coinfection with Leishmania major", J Virology 1997; 71: 3702-3709; Fassone et al, "The role of cytokines in the pathogenesis and management of aids-related lymphomas", Leuk Lymphoma 2000;38:481-488); transplantation (Li et al, "Systemic administration of anti-interleukin-10 antibody prolongs organ allograft survival in normal and presensitized recipients"; Transplantation 1998; 66: 1587-1596; Jiang et al, "Inhibition of IL-10 by FK 506 may be responsible for overcoming ongoing allograft rejection in the rat", Transplantation Proceedings 1999;31:1203-1205.), burn injury - induced immune suppression (Lyons et al, "Protective effects of early interleukin 10 antagonism on injury-induced immune dysfunction", Arch Surg 1999;134:1317-1323; discussion 1324); rheumatoid arthritis (al-Janadi et al, 1996, ibidem). Suppression of IL-10 might also be useful for cellular vaccine development (Igietseme et al, "Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development", J Immunol 2000;164:4212-4219). In many of these potential indications, the therapeutic potential of IL-10 suppression or antagonism has been supported by the efficacy of anti-IL-10 antibodies, antisense oligonucleotides specific for IL-10, and low molecular weight compounds, e.g. as described in WO 93/02693; WO 94/04180; WO 97/38695; DE 19529026; WO 99/67388). Since IL-10 inhibits cytokine synthesis in Th1 T cells (Fiorentino et al, 1991, J.Immunol. 146: 3444-3451); suppression of IL-10 might be expected to restore the production of Th1 cytokines, such as IFNγ, and thereby ameliorate allergic, Th-2-mediated diseases, such as asthma and atopic dermatitis. In fact, IFNγ is used therapeutically in the treatment of atopic dermatitis (Schneider et al, "Long-term therapy with recombinant interferon-gamma (rIFN-γ) for atopic dermatitis", Ann Allergy, Asthma, Immunol 1998;80:263-268). Furthermore, e.g. since the PPARγ agonist troglitazone is clinically effective in the treatment of psoriasis (Ellis CN et al, Arch Dermatol 2000,136: 609-616), the suppression of IL-10 might explain the clinical efficacy in this disorder.

Regulators, e.g. inhibitors, of IL-10 production are therefore indicated for use as pharmaceuticals, e.g. in the treatment of diseases associated with IL-10 production, e.g. including treatment or prevention of systemic lupus erythematosus, infectious diseases, AIDS (especially related lymphomas), cancer, transplantation, burn injury - induced immune suppression, psoriasis, rheumatoid arthritis, diseases where high Th1 responses are therapeutic (e.g. cellular vaccine development as a therapeutic), allergic diseases.

In one aspect the present invention provides the use of a PPARγ-agonist in the production of a medicament in the prevention or treatment of diseases associated with IL-10 production, e.g. including treatment or prevention of systemic lupus erythematosus, infectious diseases, AIDS (especially related lymphomas), cancer, transplantation, burn injury - induced immune suppression, psoriasis, rheumatoid arthritis, diseases where high Th1 responses are therapeutic (e.g. cellular vaccine development as a therapeutic), allergic diseases.

A PPARγ-agonist may be administered according, e.g. analogously, to known PPARγ-agonists, e.g. troglitazone, pioglitazone, rosiglitazone; e.g. in similar dosage ranges and in the form of similar pharmaceutical compositions.

Test systems for identifying a PPARγ-agonist and test systems for identifying an inhibitor of IL 10 production are known or may be provided according to, e.g. analogously to, a method as conventional.

In the following examples all temperatures are in degree Celsius.
Abbreviations:

BSA:	Bovine serum albumin
DMSO:	N,N-dimethylsulphoxide
EC_{50}:	"effective concentration 50"; concentration at which 50% of maximum induction occurs
ELISA:	Enzyme-linked immunosorbent assay
IC_{50}:	"inhibitory concentration 50"; concentration at which 50% inhibition occurs
IL-10:	Interleukin-10
LPS:	Lipopolysaccharide
mAB:	monoclonal antibody
PBS:	phosphate-buffered saline

PMA: Phorbol 12-myristate 13-acetate

PPAR: peroxisome-proliferator-activated receptor

RT: room temperature

U937: human monocytic cell line

Example

Testing of troglitazone, pioglitazone, rosiglitazone and the compound KRP-297 as inhibitors of IL-10 production

General procedure

LPS from E.coli (Serotype 055:B5) obtained from Sigma (Catalog # L-2880) is dissolved in cell culture medium at 1 mg/ml and stored as aliquots at –20°. PMA obtained from Sigma (Catalog # P-8139), is dissolved in DMSO at 10 mg/ml and stored as aliquots at –20°C. The PPARγ agonists troglitazone (CAS# 97322-87-7), pioglitazone (CAS# 111025-46-8), rosiglitazone (CAS# 122320-73-4) and the PPARγ/PPARα dual agonist KRP-297 (CAS# 213252-19-8) are used as test compounds. U937 cells are cultured in RPMI 1640 (Gibco-BRL, Paisley, UK), supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 2 mM glutamine, 0.1 mg/ml streptomycin, and 100 units/ml penicillin (Gibco). Cells from exponentially growing cultures (5-8 x 10^5 cells/ml) are centrifuged (1250 rpm [200xg]; 10 min), resuspended in fresh medium and counted. Cells are then diluted with medium to 2 x 10^5 cells/ml, supplemented with 10 ng/ml PMA and added to 96-well flat-bottomed cell culture microtiter plates (Nunc, Roskilde, Denmark; Catalog # 167008) at 20000 cells/0.1 ml/well to columns 1-11. The solvent control and each compound concentration are tested in triplicate (i.e. 3 wells per concentration) unless otherwise indicated. For compound testing, 4-fold concentrated solutions of LPS and test compounds are added (0.05 ml per well of each). Column 2 (B-G) contains the solvent control. LPS is added to the top 3 wells (B-D; "High Control") and not to the bottom 3 wells (E-G; "Low Control") of this column. Otherwise, LPS is added to columns 2 – 11 (Rows B-G). The final volume per well is 0.2 ml/well.

Test compounds (when not directly soluble in cell culture medium) are usually dissolved in DMSO at ≥ 1000-fold the highest, final concentration in the assay. Serial dilutions of these concentrated compound solutions are made in DMSO, which are subsequently diluted 250-fold into cell culture medium to generate 4x-concentrated compound solutions containing a DMSO concentration of ≤ 0.4% (v/v). The subsequent addition of these concentrated compound solutions to the cell suspensions in the microtiter plates as described above (i.e. a further 4-fold dilution of compound and DMSO) results in a uniform, final DMSO concentration of ≤ 0.1 % (v/v).

After the initial 2-day incubation with PMA at 37°C/5% CO_2 to allow for differentiation, test compounds are added as described above, and incubated for 2 hours before the addition of LPS. After a further incubation for 24 hours, the plates are centrifuged (1250 rpm; 10 min) in a Beckman GS-6KR centrifuge. The supernatants are collected from each well (~0.15-0.18

ml/well) and stored at –20° until analyzed for IL-10 via enzyme-linked immunosorbent assay (ELISA).

For the IL-10 ELISA the coating antibody (Pharmingen; Catalog # JES3-9D7) is dissolved in PBS and stored as aliquots at +4°C or –20°C. Frozen aliquots are generally thawed and used up in one experiment. Carbonate-bicarbonate buffer capsules (Sigma; Catalog # C3041) are dissolved in distilled water (1 capsule in 100 ml) to generate a 0.05 M bicarbonate buffer. Wash buffer consists of PBS containing 0.5% Tween 20 (Serva; Catalog # 37470). Blocking buffer consists of PBS containing 5% Tween 20 and 1% casein hydrolysate (Oxoid; Catalog # L41). IL-10 is obtained from Pharmingen (Catalog # 19701V) and stored as 20 ng/ml aliquots in culture medium at –20°C. The biotinylated detection antibodies for IL-10 (Pharmingen; Catalog # 18562D) is stored at +4°. The avidin alkaline phosphatase conjugate ExtrAvidin is purchased from Sigma (Catalog # E-2636). The Alkaline Phosphatase Substrate Kit is obtained from BioRad (Catalog # 172-1063), which consists of diethanolamine buffer concentrate and p-nitrophenyl phosphate tablets. 2 ml of the diethanolamine buffer concentrate are added to 8 ml of distilled water and used to dissolve two p-nitrophenyl phosphate tablets. This provides enough substrate solution for one complete 96-well plate. 2 N NaOH is purchased from Merck.

96-well microtiter plates (Maxisorp F96 Immuno Plates; Nunc; Catalog # 442404) are coated with anti-IL-10 mAB at 3 µg/ml (0.05 ml/well) overnight at RT. Coating antibody is then removed and the plates are washed three times (0.2 ml/well) with wash buffer followed by the addition of blocking buffer (0.25 ml/well) for one hour at RT. The plates are then washed again three times (0.2 ml/well) with wash buffer. 0.05 ml of the supernatants and the standards (2x-concentrated) are added per well to the plates followed by 0.05 ml/well of the biotinylated detection antibody (in culture medium; final concentration in the plates: 0.25 µg/ml, respectively). Well A1 is reserved for the blank (0.05 ml culture medium) and the rest of the top row (row A) is used for the standards (single determinations), which are: 0.005, 0.010, 0.020, 0.039, 0.079, 0.157, 0.313, 0.625, 1.25, 2.5, and 5 ng/ml (final concentrations in the plates; diluted from stocks with culture medium). Otherwise, the ELISA plates corresponds 1:1 to the cell cultures plates, regarding the positions of the samples and controls. After a 2-hour incubation at RT, the plates were washed three times (0.2 ml/well) with wash buffer followed by the addition of ExtrAvidin (diluted 1:1000 in blocking buffer; 0.05 ml/ml) for 2 hrs at RT. The plates are then washed three times (0.2 ml/well) with wash buffer followed by the addition of substrate solution (0.1 ml/well). The plates are incubated at RT until color development is sufficient for photometric detection (generally approx. 15 min).

The reaction is stopped by the addition of 2N NaOH (0.05 ml/well). The optical densities are determined in a microtiter plate reader (SLT 400 ATC, SLT Systems; Salzburg, Austria) at 405 nm with a reference wavelength of 492 nm.

Data Analysis

The absorbance data (ASCII files) from the ELISA plates are copied and pasted into preformulated Excel™ spreadsheets. The value in A1 is used as the blank and subtracted from the other values. The optical densities of the standards are fitted using the Excel add-on program XLfit™ (ID Business Solutions, Guilford, Surrey, UK) to the 4-parameter logistic equation (Model 205):

$y = A + ((B-A)/(1+((C/x)^D)))$

wherein x = concentration values of the standards and y = optical densities of the standards. The fitted parameters are:

A: bottom plateau of the curve; B: top plateau of the curve; C: x value at the middle of the curve; D: slope factor (also known as the Hill coefficient).

The fitted 4-parameter logistic equation is used to calculate the concentration of cytokine in each well from the optical density. Any value falling below the lowest standard concentration is set to zero. The calculated concentrations are multiplied by the dilution factor and the average and standard deviations of the triplicate determinations are calculated. The dose-response curves for each test compound are then fitted via Xlfit using the same 4-parameter logistic equation (Model 205) as described above.

The IC_{50} for the assay is defined as the midway point between the value of the solvent control containing LPS ("High Control") and the value of the solvent control without LPS ("Low Control"). This midway point (i.e. the x value thereof) is calculated by XLfit via the fitted logistic equation for the calculated cytokine concentration data. Another definition of the IC_{50} is the parameter C (x value at the middle of the fitted curve = midway point between the fitted top and bottom plateaus).

Results

TABLE 1 below summarizes the results obtained. As shown in TABLE 1 all of the known PPARγ agonists tested inhibited LPS-induced IL-10 secretion from PMA-differentiated U937 cells with IC_{50}s ranging from submicromolar to nanomolar concentrations. The rank order of activity correlates reasonably well with the published activity of these compounds in a

PPARγ-assay, but not in a PPARα-reporter gene assay (Willson et al, J Med Chem 2000; 43:527-550), indicating that PPARγ is the molecular target.

TABLE 1

	IC_{50} (μM)		EC_{50} (μM)	
Test compounds	IL-10 (Midway Pt.)	IL-10 (Parameter C)	PPARγ-GAL4 Transactivation[§]	PPARα-GAL4 Transactivation[§]
Rosiglitazone	0.037	0.014	0.043	ia
KRP-297	0.13	0.21	0.083	0.85
Pioglitazone	0.35	0.23	0.58	ia
Troglitazone	0.59	0.83	0.55	ia

[§] From Willson et al 2001; ia = inactive at 10 μM

Patent Claims

1. Use of a PPARγ-agonist in the production of a medicament for the prevention or treatment of diseases associated with IL-10 production.

2. Use according to claim 1 for the prevention or treatment of systemic lupus erythematosus, infectious diseases, AIDS (especially related lymphomas), cancer, transplantation, burn injury - induced immune suppression, psoriasis, rheumatoid arthritis, diseases where high Th1 responses are therapeutic, allergic diseases.

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Application No: GB 0108087.8
Claims searched: 1 and 2

Examiner: Dr William Thomson
Date of search: 10 October 2001

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Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.S): (Not searched)

Int Cl (Ed.7): A61K 31/427, 31/4427

Other: ONLINE: CAS-ONLINE, EPODOC, JAPIO & WPI

Documents considered to be relevant:

Category	Identity of document and relevant passage		Relevant to claims
X	WO 00/62766A2	(SMITHKLINE BEECHAM PLC) See whole document, in particular page 2, line 8 to page 3, line 36, page 4, lines 4-20 and claims 1-11	1 and 2
X	WO 00/30628A2	(GENENTECH INC)) See whole document, in particular page 3, lines 8-20, page 5, lines 27-31, page 9, lines 20-32 and claims 1-13	1 and 2
X	US 5981586	(PERSHADSINGH) See whole document, in particular column 2, line 66 to column 3, line 30, Examples 3 and 6 and claims 1, 3 and 6	1 and 2
X	US 5925657	(SEED ET AL) See whole document, in particular column 1, lines 38-44 and 50-55, column 2, lines 1-7 and claims 1-8	1 and 2
A	Chemical Abstract No 135:56409 & Y.Azuma et al, Biochem.Biophys.Res.Commun., (2001), 283(2), 344-346 See abstract		

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

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Claims searched: 1 and 2

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Category	Identity of document and relevant passage	Relevant to claims
X	Chemical Abstract No 135:14055 & H.Ghanim et al, J.Clin.Endocrinol., (2001), 86(3), 1306-1312 See abstract	1 and 2

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Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.